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ORIGINAL ARTICLE

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Human skeletal muscle energy metabolism: when a physiological model promotes the search for new technologies

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Abstract The idea that muscle function and, in fact, the function of all living tissues may be described by physical laws appeared in the second half of the 19th century. During this period, Helmoltz (*Über die Erhaltung der Kraft*, 1847) showed that the principle of energy conservation may be applied to living systems. In the field of exercise physiology, this idea has been subsequently developed by Rodolfo Margaria's (1901–1983) School and the mathematical formalization of the theory has succeeded in the bioenergetic model. During the last 20 years methodical and critical study of the bioenergetic model has been carried out by Paolo Cerretelli, one of the most important heirs of Rodolfo Margaria's School. Original results and technological developments have been generated by his activity and many young scientists have been educated in this approach. The present paper wants to present the modern history of the bioenergetic model and is dedicated to Paolo Cerretelli on the occasion of his seventieth birthday.

Keywords Magnetic resonance imaging · Near infrared spectroscopy · ^{31}P-NMR spectroscopy · Review · Ultrasonography

Once upon a time there was…

When I met Paolo Cerretelli for the first time, I found in front of me a convinced believer in the efficacy of the interdisciplinary approach applied to the study of human physiology. This is probably the reason why, in October 1985, he decided to engage me in the Department of Physiology as a young theoretical physicist. This date was also the starting point of the present scientific history. At that time, my abstract mathematical language was completely incomprehensible to medical doctors. Even worse, medical jargon and biological concepts appeared to me as coming from another planet. These were of course the joys of interdisciplinarity. Given these exciting starting conditions, the task of our group was to study human skeletal muscle metabolism by magnetic resonance spectroscopy. As a theoretical physicist, I abhorred the idea of performing measurements without a mathematical theory to prove and the question haunting my thoughts was: does a mathematical formulation describing skeletal muscle energy metabolism and having any practical utility actually exist?

Of course, the young naïve assistant realized very soon that Paolo Cerretelli was one of the leaders among the heirs of the "School of Margaria (1901–1983)". in The early 1960s Rodolfo Margaria's School had generated a theory describing skeletal muscle metabolism that was then translated over the course of the years into a mathematical form. For simplicity, from now on I will call the formalized form of the theory: the "bioenergetic model". In our laboratory, the bioenergetic model has become the central point around which an interdisciplinary approach to muscle energetics has been developed. As you will see, the study of the bioenergetic model has stimulated the development of new interesting experimental protocols and instrumentation.

The present paper is not a comprehensive review of skeletal muscle energetics but has the aim of highlighting some original features of the research activity promoted by Paolo Cerretelli during his period at the University of Geneva. The following text is divided into a series of short examples each of which highlights a particular utilization of the bioenergetic model. These may illustrate a theoretical derivation, an experimental application or the requirement for the development of new methods to exploit or test the potentialities of the theory.

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The bioenergetic model: defining constraints on the energetic sources

The idea that muscle function and, in fact, the function of all living tissues may be described by physical laws appeared in the second half of the 19th century (for an extended historical and scientific review on bioenergetics see di Prampero 1981). During this period, Helmoltz (1847) showed that the principle of energy conservation may be applied to living systems. Based on this principle, many experimental studies trying to find muscle "energy yielding molecules" were performed. By the beginning of 20th century, this domain of research had also generated the fundamental theory of muscle contraction proposed by Hill and Meyerhof (Hill 1965). These studies and other work led to the basis of muscle bioenergetics. Today, one can say that the main mechanisms of muscle function have been clarified and, in particular, that the main energy sources have been identified.

To describe the metabolic processes occurring in the muscle cell, it is necessary to take into account hundreds of biochemical reactions. The practical consequence of this is that the results obtained in the last 20 years by the scientists involved in the modelling of the metabolic control (Fell 1997) are extremely difficult to apply when dealing with human subjects or patients. Thus, to solve this problem, it is necessary to define a "meta-theory" that highlights only the essential characteristics of muscle function. Moreover, it is crucial that the parameters appearing in the theory are measurable in vivo. The bioenergetic model proposed by the Margaria's School represents one of these possible "meta-theories", embodying the behaviour of the underlying biochemical reactions in a coherent way.

In this context, two principles may intuitively summarize the bioenergetic approach. The first can be expressed as follows: the energy utilized by the muscles during work (and rest) is stored as ATP (Lundsgaard 1930). Thus, to quantify the total energy expenditure of the muscle (at rest or during exercise), it would be enough to measure ATP consumption. Actually, the ATP concentration is maintained constant in the muscle in the majority of situations thanks to a series of biochemical reactions that continuously resynthesize ATP. As a consequence, in the majority of cases, ATP measurements alone do not provide much information regarding normal or pathological muscle states. For this reason it is necessary to refine the first principle with a second statement – the energy utilized during muscular activity (and rest) mainly comes from three sources: aerobic glycolysis, anaerobic glycolysis and the Lohmann reaction (di Prampero 1981). The sum of the energy produced by these three sources yields the total energy expenditure of the system. Now, the first principle (and experimental measurements) implies that aerobic glycolysis, anaerobic glycolysis and the Lohmann reaction must continuously replace the ATP hydrolysed during muscle contraction. Studies on bioenergetics (di Prampero 1981; Kushmerick 1983; Kemp and Radda 1994) have shown that to evaluate the energy produced by these three sources, and to obtain an estimation of the number of ATP molecules hydrolysed for a given muscular activity, it is sufficient to measure the concentration changes of three key molecules. These observations can be summarized in formal terms as:

$$\dot{E}_{\text{work}} + \dot{E}_{\text{basal}} = \left[\dot{\text{ATP}}\right] + \alpha\left[\dot{\text{PCr}}\right] + \beta\left[\dot{\text{La}}\right] + \gamma\left(\left[\dot{\text{O}}_2\right]_{\text{work}} + \left[\dot{\text{O}}_2\right]_{\text{basal}}\right) \quad (1)$$

where E_{work} (the dot is, as usual, the time derivative) represents the instantaneous energy consumption of the muscle per unit time during exercise (in ATP concentration units, mM s^{-1}) and [ATP], [PCr], [La] and [O_2] are the concentrations of ATP, phosphocreatine (PCr), lactate (La) and oxygen (O_2), respectively. The term $\dot{E}_{\text{basal}} = \gamma\left[\dot{\text{O}}_2\right]_{\text{basal}}$ represents the basal metabolism. The first term on the right-hand-side of Eq. 1 contributes only in extreme conditions when the muscle cannot succeed in maintaining a constant ATP concentration (e.g. fatigue). (α, β and γ represent the number of ATP molecules produced by one molecule of [PCr], [La] and [O_2], respectively.)

Of course, α[PCr], β[La] and γ[O_2] yield the ATP produced by the Lohmann reaction, anaerobic glycolysis and aerobic glycolysis, respectively. The essential point here is that the molecules appearing in Eq. 1 are all measurable, directly or indirectly, in humans.

It is necessary at this point to define the efficiency (η) of the ATP utilization to develop mechanical work:

$$\eta = \frac{\dot{w}}{\dot{E}_{work}} \quad (2)$$

where $\dot{w}$ represents the mechanical power (J s^{-1}). Intuitively, η can be viewed as the number of ATP molecules necessary to produce a given amount of mechanical energy w.

Equation 1 may be considered as a metabolic constraint that should enable one to calculate the "missing" terms, i.e. the terms that in some protocols cannot be measured experimentally. It must be noticed, that Eq. 1 holds for one muscle fibre or for the whole tissue because, in the latter case, it represents the total energy of the system (the sum of all the fibres). This last observation suggests that bioenergetics allows one to integrate different complexity levels of the muscular system. For example, any theory describing the mode of recruitment of muscular fibres should satisfy the constraints imposed by Eq. 1. Thus, the bioenergetic approach itself can be regarded as a novel "instrument" for the modelling of neurophysiological phenomena. The validity and the limits of Eq. 1 are well established in normothermia and for different pathological conditions (see reviews mentioned above). However, very little is known about the behaviour of the different terms in Eq. 1 and on the efficiency parameters during muscle hypo- or hyperthermia in humans.

Thus, to summarize one can combine Eqs. 1 and 2 and explicitly introduce the temperature (T) dependence as follows:

$$\frac{\dot{w}}{\eta(T)} + \dot{E}_{\text{basal}}(T) = \left[\dot{\text{ATP}}(T)\right] + \alpha(T)\left[\dot{\text{PCr}}(T)\right] + \beta(T)\left[\dot{\text{La}}(T)\right] + \gamma(T)\left\{\left[\dot{\text{O}}_2(T)\right]_{\text{work}} + \left[\dot{\text{O}}_2(T)\right]_{\text{basal}}\right. \quad (3)$$

where $\dot{w}$ represents the mechanical power developed by a subject.

For many years Eq. 3 has represented Paolo Ceretelli's "vision" and he has stimulated and encouraged his young colleagues to participate in exploring its ramifications. As is the case for any model, this approach is not perfect and one day it may well be discard or be included in a better theory; however, as we will discover in the following sections, Eq. 3 has been the source of many ideas, new experimental protocols and international collaborations. The necessity of measuring the parameters appearing in Eq. 3 has also been the driving force behind the adoption of new measurement technologies such as nuclear magnetic resonance (imaging and spectroscopy) or near infrared spectroscopy. Moreover, we will also see that new technical developments have been promoted by the study of the bioenergetic model applied to human energy metabolism.

Access to anaerobic alactic metabolism: ^{31}P-NMR spectroscopy

This first example aims to show how the bioenergetic model has allowed us to predict some key properties of the dynamic behaviour of the energy-yielding sources on the bases of data measured only at steady state. Let us consider an aerobic rest-to-work exercise transient. In this case, during the exercise interval, Eq. 3 may be written as:

$$\dot{E}_{\text{work}} + \dot{E}_{\text{basal}} = \frac{\dot{w}}{\eta} + \dot{E}_{\text{basal}} = \alpha\left[\dot{\text{PCr}}\right] + \gamma\left[\dot{\text{O}}_2\right]_{\text{work}} + \gamma\left[\dot{\text{O}}_2\right]_{\text{basal}} \quad (4)$$

where the variable T has been omitted because it is assumed to be constant. The terms $\left[\dot{\text{ATP}}\right]$ and $\beta\left[\dot{\text{La}}\right]$ are negligible since, due to the kind of exercise, no *net* energy comes from anaerobic glycolysis or from the ATP energy stores. Taking into account the fact that basal metabolism is usually considered to yield a constant energy contribution and that $\dot{w}$ is the measured variable (mechanical power in W), Eq. 4 may be simplified as:

$$\frac{\dot{w}}{\eta} = \alpha\left[\dot{\text{PCr}}\right] + \gamma\left[\dot{\text{O}}_2\right]_{\text{work}} \quad (5)$$

Under these physiological conditions, the efficiency η can be considered as constant. At rest, $\left[\dot{\text{PCr}}\right] = 0$, because [PCr] is obviously constant. At the onset of the exercise $\left[\dot{\text{O}}_2\right]_{\text{work}} = 0$ and the energy comes exclusively from the anaerobic alactic metabolism (PCr). So, for a given constant exercise intensity $\dot{w}$, once one has measured one of the two parameters on the right-hand-side of Eq. 5, the second may be simply derived by subtraction. It follows that, for an aerobic exercise transient, $\gamma\left[\dot{\text{O}}_2\right]_{\text{work}}$ is the "mirror image" of $\alpha\left[\dot{\text{PCr}}\right]$, independent of the time course of the relationship. It is of note that the fundamental concept relating, in a energy balance equation, high-energy phosphates and oxygen consumption during exercise was first introduced by Margaria 70 years ago. Subsequently, this approach was further developed, both theoretically and experimentally by his School (see e.g. Margaria et al. 1933; di Prampero and Margaria 1968).

At this point, more information may be obtained from Eq. 5 but to do so, one key requirement is the capability of measuring intramuscular PCr and $\left[\dot{\text{O}}_2\right]_{\text{work}}$ repeatedly and for different work load levels. In the 1970s, the advent of ^{31}P-NMR spectroscopy allowed us to measure [PCr] non-invasively in human muscle and made it possible to repeat measurements as many times as necessary. The pioneering work of the Oxford group opened up the unique opportunity of testing the bioenergetic model and following metabolic transients (Hoult et al. 1974). This was of course not possible previously with the standard technique of biochemical analysis of biopsy samples. This was one of the reasons why Paolo Cerretelli was amongst the first enthusiastic adopters of this exciting technology.

Thanks to this new approach, it has been possible to demonstrate that during an aerobic exercise [PCr] reaches a steady-state (constant) value, i.e. $\left[\dot{\text{PCr}}\right] = 0$ (Binzoni et al. 1992). Moreover, it has also been shown that [PCr] at a steady state is linearly related to the workload level $\dot{w}$ (Binzoni et al. 1992). This result has an important consequence and demonstrates the predictive power of Eq. 5. ln fact, if combined with Eq. 5, it necessarily implies that the time constant (τ) of the $\alpha\left[\dot{\text{PCr}}\right]$–time curve at the onset of exercise is constant (see Fig. 1), i.e. independent of $\dot{w}$ (Binzoni and Cerretelli 1994). The parameter τ has been measured at a stable value of 23.4 s and intuitively represents the speed at which the oxidative metabolism is started (Binzoni et al. 1992). Thus, thanks to Eq. 3, steady-state measurements may be utilized to infer properties (in the present case τ) concerning the dynamic evolution of the system. The above results may also be seen in a more general context when the [PCr] vs. time behaviour is not necessarily an exponential, but this is not a matter for the present paper.

Another interesting result follows from the constraints imposed by Eq. 5 and the linear relationship existing between [PCr] at steady state and $\dot{w}$. In fact, as originally pointed out by di Prampero and Margaria (1968), the slope of the [PCr]–$\dot{w}$ line uniquely defines τ (Binzoni and Cerretelli 1994). The larger the slope, the larger is τ, i.e. the slower is the onset of aerobic metabolism. Thus, by measuring the [PCr]–$\dot{w}$ slope of different muscle types, it should be possible to obtain information concerning their aerobic capacity during metabolic transients. Again this

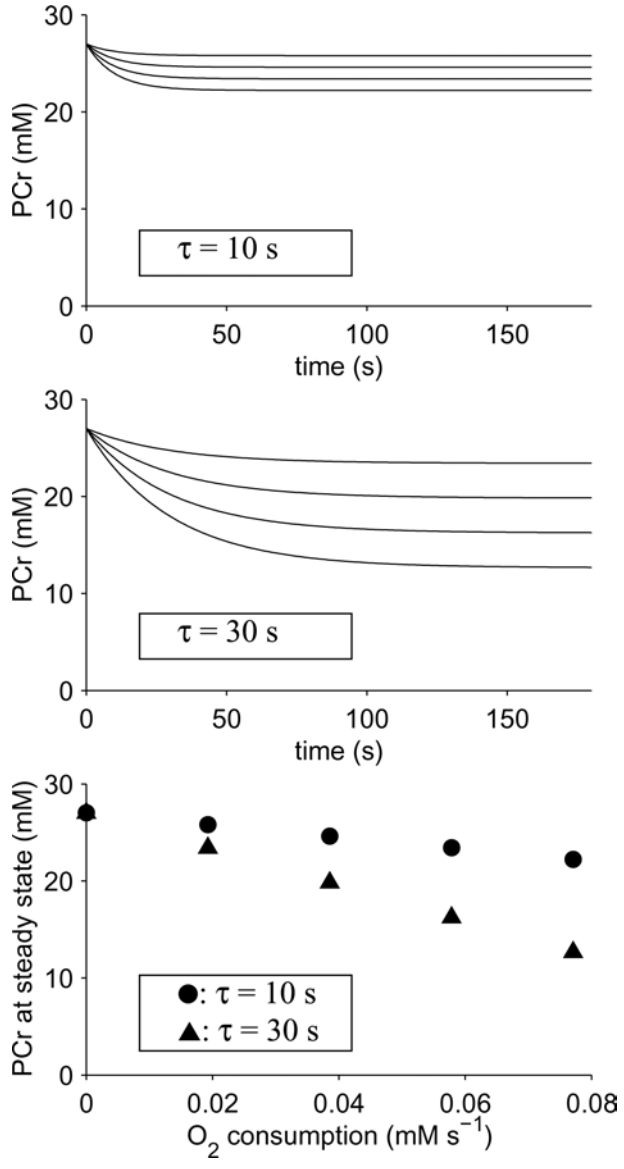

Fig. 1 Time course of PCr concentration as predicted from the bioenergetic model (Eq. 5), during a constant load aerobic exercise, for $\left[\dot{O}_2\right]$ equal to 0.0193, 0.0386, 0.0578 and 0.0771 mM s^{-1} from *top* to *bottom*; ($\alpha = 1$; $\gamma = 6.2$; onset of the exercise at $t = 0$ s). For didactical reasons the PCr kinetic is considered exponential with a time constant $\tau = 10$ s (*top panel*) or 30 s (*central panel*). The basal $\left[\dot{O}_2\right]$ was set to 0 mM s^{-1} and PCr at rest = 27 mM (see text). *Bottom panel*: PCr values at steady state (flat part of the PCr–*t* curves) as a function $\left[\dot{O}_2\right]$. (*Closed circles* Values for $\tau = 10$ *top panel*, *closed triangles* values for $\tau = 30$ *central panel*)

information is obtained only by considering data obtained at a steady state. Figure 1 summarizes graphically the findings presented above.

Temperature measurements and basal oxidative metabolism: ^{1}H-MRI

Let us now come back to the general expression of the bioenergetic model (Eq. 3), where T appears explicitly. The dependence of oxidative metabolism on T is not very well known in humans, especially when the muscle T changes are local. The lack of experimental data is due to the difficulty of experimentally determining the relationship between local intramuscular $\left[\dot{O}_2(T)\right]_{basal}$ and T in humans. Moreover, large T gradients may exist between superficial and deep muscle regions and this may lead to a "mix" of different local metabolic activities if the measurements cover large tissue volumes. The consequence is the loss of precise physiological information. In the present section, I will discuss a particular approach to this problem, using ^{1}H magnetic resonance imaging (^{1}H-MRI). The physiological aim will be to study some aspects of the influence of local T changes on resting muscle metabolism. In this case, the experimental conditions may be summarized by simplifying Eq. 3 as:

$$\dot{E}_{basal}(T) = \gamma(T)\left[\dot{O}_2(T)\right]_{basal} = \gamma(T)\left\{\left[\dot{O}_2(T)\right]_{metabolic} + \left[\dot{O}_2(T)\right]_{heat}\right\} \quad (6)$$

where the basal metabolism has been split into two terms, the first of which, $\gamma(T)\left[\dot{O}_2(T)\right]_{metabolic}$, represents the oxidative component involved in ATP production. The second term, $\gamma(T)\left[\dot{O}_2(T)\right]_{heat}$ is the fraction of $\left[\dot{O}_2(T)\right]_{basal}$, expressed in ATP units, that contributes to heat production in resting muscle, which obviously enough requires oxidative energy. At the time when these studies were performed, the problem was to measure the parameters appearing in Eq. 6, since no method existed to measure local oxygen consumption. For this reason attention was focused on the term $\left[\dot{O}_2(T)\right]_{heat}$ and the main idea was to measure intramuscular spatially localized heat fluxes to obtain indirect information on $\left[\dot{O}_2(T)\right]_{heat}$ (Binzoni et al. 1995). In fact, knowledge of the spatial and temporal T distribution ($T(x, y, t)$) in the muscle allows one to obtain the heat fluxes by solving the bioheat equation (Pennes 1948; Nelson 1998):

$$\rho c \frac{\partial}{\partial t} T(x,y,t) = k\nabla^2 T(x,y,t) + q(x,y,t) \quad (7)$$

where ρ, c and k are the tissue density, the specific heat of tissue and the specific thermal conductivity, respectively. Finally, the term $q(x, y, t)$ describes the heat production rate in the muscle which is proportional to $\left[\dot{O}_2(T)\right]_{heat}$ in Eq. 6. It must be noticed that Eq. 7 takes into account the original Pennes assumption that the temperature gradient is negligible along the long axis of the forearm (Pennes 1948). This means that convective heat exchange occurs only at the capillary level (Tikuisis and Ducharine 1991) and is described by the $q(x, y, t)$ term.

In summary the protocol used was the following. The subject's legs were heated up to 42°C in a temperature-controlled bath. Then, the subject was exposed to room T (23°C) in the bore of the MRI together with supplementary cold air flow. A series of images were obtained at 50-s intervals (transverse slices) using an echo sequence. This ^{1}H-MRI sequence is T sensitive. A T

calibration of the image was obtained by means of an intramuscular microthermocouple. The image sequence was then fitted as a function of space and time using a neural network. This allowed us to obtain a numerical function describing the T distribution of a region of interest during muscle cooling, i.e. $T(x, y, t)$ for a given slice position z.

The experimental results are summarized in Fig. 2, where $q(x, y, t)$ is represented as a function of T. For simplicity, all the points are reported on the same graph independently of their time (t) or spatial location (x, y). The data reported in Fig. 2 are the first ever heat flow, $q(x, y, t)$, measurements in humans. However, this is not the end of the story. In fact, $q(x, y, t)$ contains not only the heat produced by the $\left[\dot{O}_2(T)\right]_{heat}$ component but also the heat transported by the blood. Thus, to distinguish between the two components it has become necessary to measure directly $\left[\dot{O}_2(T)\right]_{basal}$ and blood flow. Again, a new technical challenge was generated by the study of the bioenergetic model. Later in this paper a new approach will be presented.

Anaerobic alactic energy sources and temperature: ^{31}P-NMR spectroscopy

The effect of cooling or heating a biological tissue is not a simple physical phenomenon and many variables may influence the $\left[\dot{O}_2(T)\right] - T$ relationship. Changes in thermodynamic conditions, blood perfusion, enzymatic activity, the concentration of key metabolites, etc. may influence the $\left[\dot{O}_2(T)\right] - T$ curve. Actually, Eq. 3 explicitly contains some metabolites, ATP and PCr, whose concentration might indirectly influence $\left[\dot{O}_2(T)\right]$. In fact, the stoichiometric equilibrium between [ATP], [PCr] and [H^+] determines the [ADP] levels. It is well known that ADP is an important modulator of oxygen consumption (Brown 1992; Mader 2003). So, we decided to study the T concentration dependence of these molecules (Binzoni et al. 2000a). This should provide supplementary information on both the bioenergetic model and the associated regulatory mechanisms.

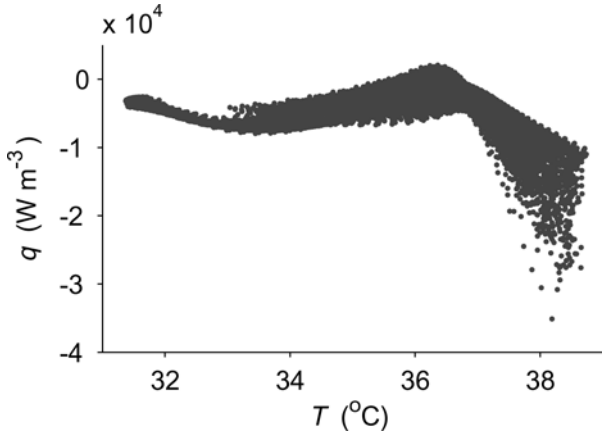

Fig. 2 Intramuscular heat flow as a function of temperature in the human vastus lateralis. The measurements were performed during muscle cooling. Negative q sign corresponds to heat loss, so that a decrease in q reflects an increase in heat loss (figure adapted from Binzoni et al. 1995)

The calf of a subject was cooled at different T and relative [ATP], [PCr] and [H^+] values were obtained by ^{31}P-NMR spectroscopy. Intramuscular T was measured non-invasively using a zero-heat-flow (ZHF) probe (Fox and Solman 1971; Fox et al. 1973; Togawa 1985), the ZHF being combined with the ^{31}P-NMR radio frequency coil to observe the same region of interest. Of course, care was taken to eliminate T-induced "physical" effects that could potentially introduce artefacts into the concentration measurements assessed by ^{31}P-NMR spectroscopy.

Figure 3 shows the results obtained. It is clear that [ATP], [PCr] and [H^+] change as a function of T. Moreover, the calculated [ADP] decreases with decreasing T. This means that mitochondria are theoretically less "stimulated" at low T; in addition one would also expect that $\left[\dot{O}_2(T)\right]$ should decrease monotonically with [ADP]. Thus, if one takes into account also the fact that low T should "slow down" the oxidative metabolism by a simple thermodynamic effect, the overall result should be a decrease in $\left[\dot{O}_2(T)\right]$ with decreasing T. In the light of these considerations, the data of Fig. 2 show clearly that the fraction of heat transported directly by the blood must be the main determinant of the $q(x, y, t)$–T curve; i.e. the metabolic heat component seems to be completely masked by the heat transported by convection. Of course, this conclusion had to be proven experimentally and this was the reason that motivated the realization of the studies presented in the next sections.

Access to the tissue oxygen consumption: near infrared spectroscopy

Near infrared spectroscopy (NIRS) is the only existing technique allowing one to quantify non-invasively the local oxygen consumption of small quantities (a few millilitres) of human muscle. The utilization of NIRS for $\left[\dot{O}_2\right]$ measurements was introduced by Cheatle et al. (1991) and De Blasi et al. (1993) (for a review on NIRS applications for muscle studies, see e.g. Ferrari et al. 1997). Thus, from the beginning, it was clear for Paolo Cerretelli's team that NIRS might be the solution to the problem of $\left[\dot{O}_2\right]$ assessment during T changes.

Unfortunately, with the present technological development of NIRS systems, quantification can only be obtained via the use of some specific physiological manoeuvres (e.g. arterial occlusion), which considerably limits the possible physiological protocols that can be employed. In fact, NIRS measures oxy-(ΔHbO_2) and deoxyhaemoglobin (ΔHb) concentration *changes* and the $\left[\dot{O}_2\right]$ measurement method itself consists of stopping muscle blood flow using, for example, a Riva-Rocci cuff. Under these conditions, for each deoxyhaemoglobin

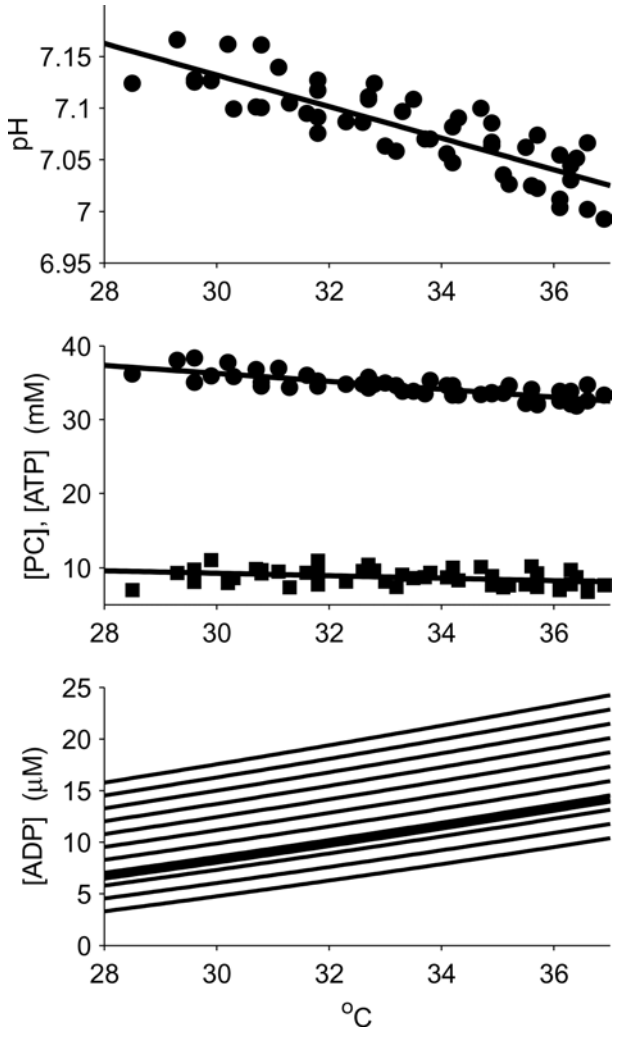

Fig. 3 *Top*: human gastrocnemius [H^+] expressed in pH units (intracellular) as a function of temperature. *Center*: human gastrocnemius [ATP] (*closed squares*) and [PCr] (*closed circles*) as a function of temperature. *Bottom*: human gastrocnemius [ADP] as a function of temperature. The values were calculated using a temperature-corrected Lohmann equilibrium equation. The *thin lines* were calculated for a total creatine concentration from 40 mM (*bottom line*) to 50 mM (*top line*), in steps of 1 mM. The *thick line* corresponds to 42.7 mM total creatine (figure adapted from Binzoni et al. 2000a)

molecule appearing in the blood, one has four O_2 molecules being utilized by the metabolizing tissue. In this manner, the ΔHb–time slope is proportional to the intramuscular $\left[\dot{O}_2\right]$ in absolute or relative values depending on the NIRS instrumentation used, i.e. on whether one is measuring absolute or relative [ΔHb] values. This method is well tested and the conditions of validity during rest and intermittent isometric exercise have been defined (Van Beekvelt et al. 2001a, 2001b, 2002). All these methods are based on the hypothesis that the obtained ΔHb–time curves are linear during the first 30–40 s, both in the muscle at rest and during a rest-work transient (i.e. neglecting the $\left[\dot{O}_2\right]$ transient phase at the onset of exercise). The above hypothesis becomes more difficult to satisfy when performing an isotonic exercise. In fact, using again the bioenergetic model, it has been demonstrated (Binzoni et al. 1999a) that in the case of an aerobic (isotonic) exercise transition (similar to that described above by Eq. 5 and where $\left[\dot{PCr}\right]$ and/or $\left[\dot{O}_2\right]$ versus time may reasonably be considered exponential with a time constant τ), the corresponding ΔHb–time curve is neither exponential nor linear but it must be written as:

$$[\Delta\mathrm{Hb}] = \frac{1.13}{4\gamma}\,\frac{\dot{w}}{\eta}\left\{t - \tau\left(1 - e^{-t/\tau}\right)\right\} \tag{8}$$

where all the quantities are as previously defined. This result means that with cuff ischaemia the measured [ΔHb] kinetics does not represent the $\left[\dot{O}_2\right]$ kinetics. Moreover, a delay of a few seconds in the [ΔHb]–time curve does not correspond to a delay in $\left[\dot{O}_2\right]$ but it is only due to the shape of the [ΔHb]–time curve (which is very "flat" during the first few seconds, see Eq. 8). From a practical point of view, this experimental approach is not suitable for the detection of subtle physiological features such as the presence of eventual delays in $\left[\dot{O}_2\right]$ at the onset of exercise (see Grassi et al. 2003 published in the present volume). Without the presence of the cuff, the [ΔHb] behaviour as a function of $\left[\dot{O}_2\right]$ becomes even more complex because of the simultaneous O_2 inflow with arterial blood. These technical problems need to be further clarified if one wants to use NIRS for $\left[\dot{O}_2\right]$ assessment under a wide range of physiological conditions.

Fortunately, as observed above, these problems do not appear or are negligible during rest or in pure aerobic isometric exercise at steady state (i.e. the [ΔHb]–time curve may be considered linear) and this has allowed us to finally assess $\left[\dot{O}_2\right]$ during T changes.

Oxidative metabolism and temperature: near infrared spectroscopy

We have seen in the above sections how it has been possible to measure local $\left[\dot{O}_2\right]$ in humans by NIRS. Moreover, we have also seen that the ZHF probe allows us to assess intramuscular T non-invasively. Using these two technologies, in collaboration with the Biomedical Optics Research Group of the Department of Medical Physics and Bioengineering (UCL, London), a new combined NIRS-ZHF probe has been developed (Binzoni et al. 1999b). The advantage of the combined NIRS-ZHF probe is that it permits one to measure intramuscular T and, at the same time, it actively produces a uniform T distribution in the region of interest sampled by the NIRS instrument. A suitable technical adaptation was introduced to allow the NIRS-ZHF probe to work not only in air but also during cold water immersion. With the NIRS-ZHF probe it has been possible to assess $\left[\dot{O}_2(T)\right]$ at different T during both rest and isometric contractions (Binzoni et al. 2002a). Measurements were performed during cooling of the forearm in a

temperature-controlled bath. $\left[\dot{O}_2(T)\right]$ during isometric contraction was assesses at 4% of the maximal voluntary contraction. Since both La production and changes in [ATP] can be excluded in this type of exercise, the bioenergetic model describing this protocol reduces to (see Eq. 3):

$$\frac{\dot{w}}{\eta(T)} + \mathbf{E}_{\text{basal}}(T) \approx \gamma(T)\left\{\left[\dot{O}_2(T)\right]_{\text{work}} + \left[\dot{O}_2(T)\right]_{\text{basal}}\right\} \tag{9}$$

where the sign $\approx$ means that the $\alpha\left[\text{P}\dot{\text{C}}\text{r}\right]$ contribution has been considered negligible for the present purposes. It must be noticed that when dealing with isometric exercise many authors prefer to use the term "cost" defined as:

$$\xi = \frac{\dot{E}_{\text{work}}}{P_0} \tag{10}$$

where P_0 represents the isometric force (N m^{-2}). In this case, Eq. 10 replaces Eq. 2 and Eq. 9 becomes:

$$\xi(T)P_0 + \dot{E}_{\text{basal}}(T) \approx \gamma(T)\left\{\left[\dot{O}_2(T)\right]_{\text{work}} + \left[\dot{O}_2(T)\right]_{\text{basal}}\right\} \tag{11}$$

The results of an experiment for a typical subject are reported in Fig. 4. It is interesting to compare Figs. 2 and 4. It can be seen that the behaviours of $q(x, y, t)$ and $\left[\dot{O}_2(T)\right]_{\text{basal}}$ (measurements at rest) are very different and that the convective heat contribution in $q(x, y, t)$ seems to have a major role. Surprisingly, $\left[\dot{O}_2(T)\right]$ during isometric contraction suddenly increases at low T. It is noteworthy that these results reproduce the $\left[\dot{O}_2(T)\right] - T$ curves found in isolated mitochondria (Davison 1971; Cossin and Bowler 1987). The difference between rest and isometric contraction theoretically gives $\left[\dot{O}_2(T)\right]_{\text{work}}$, and since P_0 is constant in this experiment, then from Eq. 9 it follows that:

$$\frac{\xi(T)}{\gamma(T)} \propto \left[\dot{O}_2(T)\right]_{\text{work}} \tag{12}$$

This means that $\xi(T)/\gamma(T)$ is proportional to $\left[\dot{O}_2(T)\right]_{\text{work}}$ and that $\xi(T)$ and/or $\gamma(T)$ values must change to follow the $\left[\dot{O}_2(T)\right]_{\text{work}}$ curve. This mathematical result is consistent with the fact that $\xi(T)$ seems to decrease at low T (Bottinelli and Reggiani 2000). A more exhaustive discussion on the T dependence of the bioenergetic equation may be found in Binzoni and Delpy (2001).

To conclude this section, a remark must be made on the fact that to obtain the above measurement it has been necessary to study explicitly the influence of T on the measurement technique. In fact, the NIRS signals are extremely T sensitive. Some artefacts due to the changes in the "physical" (mainly optical) properties of the tissue may be introduced during T variations. Practically, this means that, as in the case of ^{31}P-NMR spectroscopy, measurements made at one T cannot be compared directly to measurements made at a different T without a suitable data correction (Binzoni et al. 1999b). This T dependence of the NIRS signals has been exploited to build a prototype NIRS-based thermometer (Hollis et al. 2001). This area of research needs to be further investigated.

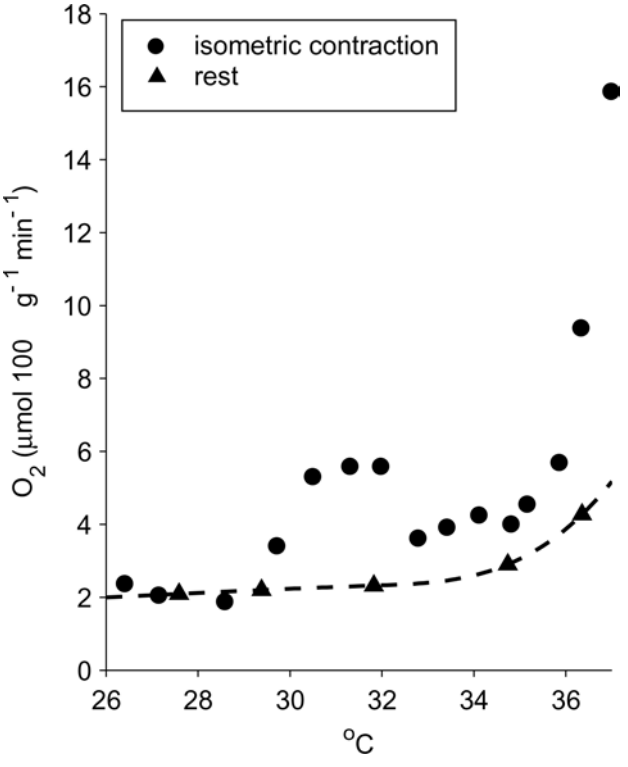

Fig. 4 Oxygen consumption curve as a function of intramuscular (forearm) temperature of a typical subject during rest (*closed triangles*) and isometric contraction (*closed circles*; figure adapted from Binzoni et al. 2002a)

Blood volume and velocity changes: an unavoidable problem

At this point, assessing intramuscular $\left[\dot{O}_2\right]$ by NIRS seemed to be a realistic issue for physiological or clinical studies. However, two major technical problems have to be solved if one wants to measure $\left[\dot{O}_2\right]$ without the need for specific physiological manoeuvers (e.g. the use of an arterial cuff). The first problem relates to the fact that during a measurement the total haemoglobin, $[\Delta Hb_{tot}] = [\Delta Hb] + [\Delta HbO_2]$ (i.e., the haemoglobin concentration per unit tissue volume), may change its concentration due to vasodilation/vasoconstriction phenomena. As a result, $[\Delta Hb_{tot}]$ changes may mask the "real" $[\Delta Hb]$ and $[\Delta HbO_2]$ metabolic variations. This may introduce an error in the $\left[\dot{O}_2\right]$ evaluation from $[\Delta Hb]$ or $[\Delta HbO_2]$ tracings or in the data interpretation (e.g. it is often impossible to distinguish which fraction of the $[\Delta HbO_2]$ variation is due to the volume and which to the metabolic change). This problem has not yet been

solved and no possible theoretical/practical solutions allowing one to distinguish metabolic from tissue haemoglobin concentration changes have been proposed until now.

Although [ΔHb_{tot}] variations may prevent precise metabolic evaluations of $[\dot{O}_2]$ measurements, it is possible to look at this technical defect as a positive feature. For this reason, Paolo Cerretelli's team decided to use the [ΔHb_{tot}] signal as a tool to investigate the microvascular bed compliance (Binzoni et al. 2000b). For this protocol a subject was lying supine on a tilting bed and [ΔHb_{tot}] was measured by NIRS on the gastrocnemius muscle. The subject was tilted head up by steps, from –10° to 75°. The results of the experiment are reported in Fig. 5 (left). From these data it has been possible to derive the microvascular compliance of the region probed by the NIRS instrument [0.086 (0.005) ml l^{-1} $mmHg^{-1}$].

Actually, in the context of the present paper, the above experiment represents a good example describing the effects of blood flow or velocity on [ΔHb] and [ΔHbO_2]. In fact, the influence of blood flow velocity on [ΔHb] and [ΔHbO_2] represents the second technical problem yet to be solved. During bed tilt, the muscle metabolism can be considered constant, while blood flow may change due to a possible vasomotor reflex (Garskell and Burton 1953). The independent change of the [ΔHb] and [ΔHbO_2] slopes, starting from ~20° (Fig. 5, right), is classically explained by the influence of the blood flow/velocity changes on haemoglobin oxygen extraction. As in the case of non-constant [ΔHb_{tot}], the reduction in blood flow/velocity has a direct influence on the individual [ΔHb] and [ΔHbO_2] values (Fig. 5, right) without the presence of a "real" metabolic change. In conclusion, this experiment highlights the necessity to monitor blood velocity if one wants to assess the muscle metabolic activity by NIRS. One possible solution to this new challenge is proposed in the next section.

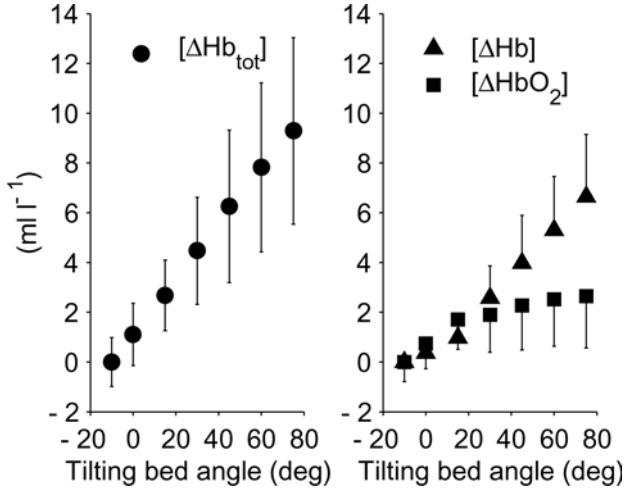

Fig. 5 Change in total blood ($[\Delta Hb_{tot}]$), deoxyhaemoglobin ($[\Delta Hb]$) and oxyhaemoglobin ($[\Delta HbO_2]$) as a function of the tilting bed angle. Each point corresponds to the mean of data from ten subjects measured at steady state at a given angle. An angle of 0° corresponds to the horizontal position; 90° to the vertical position (head up). *Vertical bars* are standard deviations

Blood velocity: near infrared laser Doppler flowmetry

The study of the bioenergetic model has demonstrated over the course of the years that it is necessary to apply different measuring techniques. In fact, each technique has allowed us to assess only some of the parameters appearing in Eq. 3. However, in order to perform simultaneous measurements of many parameters, the measurement techniques employed have to be compatible with each other. This was a key requirement that the most recent instrument developed in collaboration with the Department of Medical Physics and Bioengineering (UCL, London), and allowing blood velocity measurements, had to satisfy. In addition, considering that the velocity measurements were in principle to be utilized to extract the metabolic component from [ΔHb] and [ΔHbO_2] changes, a necessary prerequisite is that the new instrument samples exactly the same volume of tissue over which NIRS measurements are performed. This is the reason why it was decided to build a new near-infrared based laser Doppler flowmeter (LDF). This approach should in principle allow one to obtain simultaneously [ΔHb], [ΔHbO_2] and velocity data with one instrument using the same detector for all the measured parameters.

A prototype instrument demonstrated the applicability of the technique on humans (Binzoni et al. 2002b). A new algorithm for derivation of the velocity signal and based on quasi-elastic scattering theory and holding for multiple scattering has also been developed. This algorithm allows one to obtain velocity data in absolute units, and without the need for a calibration procedure as required for standard LDF instruments dedicated to skin measurements. The first ever absolute intramuscular blood velocity measurements measured on the human forearm, before, during and after arterial occlusion are reported in Fig. 6. In conclusion, knowledge of the haemoglobin concentration changes and simultaneously of blood velocity should in the future permit us to study the $[\dot{O}_2]$ component in the bioenergetic model without the need for arterial occlusion.

Muscle architecture and metabolism: ultrasonography

Before finishing this paper it is worth noting that the relationship existing between the parameter $\dot{w}$, appearing in Eq. 3, and the energy-yielding metabolites depends strongly on the anatomical structure defined by the muscle-bone-joint unit. Actually, $\dot{w}$ represents external mechanical power produced by the muscle often through a lever arm (bones). It is clear that the work supplied by the muscle, for a given $\dot{w}$, depends on muscle cross-sectional area, bone length, etc. Thus care must be taken when comparing, for example, different subjects.

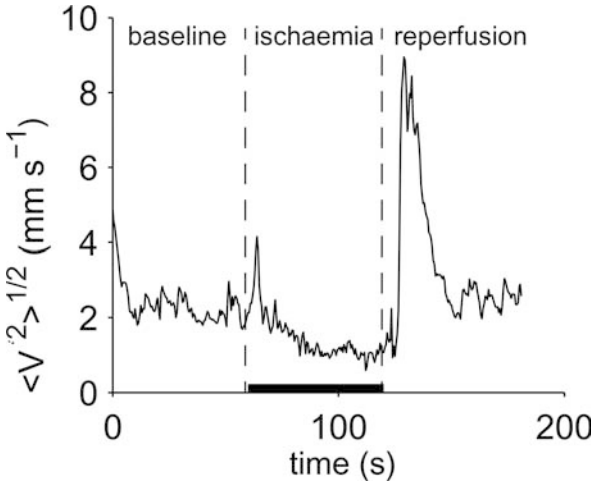

Fig. 6 Root mean square blood velocity as a function of time (human forearm) before, during and after arterial occlusion. The measurement technique (near infrared laser-Doppler flowmetry) allows one to observe small vessels (e.g. arterioles, capillaries) in few centimetres cubed of tissue

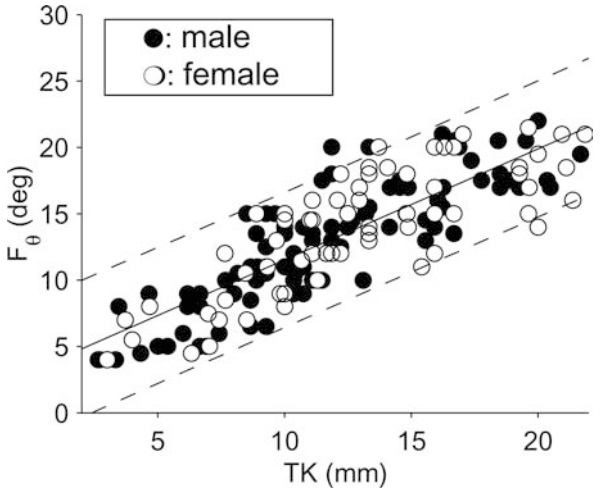

Fig. 7 Human gastrocnemius medialis pennation angle as a function of muscle thickness. The age of the subjects was distributed in the interval 0–65 years (figure adapted from Binzoni et al. 2001)

Moreover, internal muscle architecture may also influence the relationship between $\dot{w}$ and energy-yielding metabolites, linked in the bioenergetic model by the parameter η. In fact, skeletal muscle may be pennated, i.e. the direction of the muscle fibres may not be parallel to the developed force. Practically, this means that the relationship between w and energy-yielding metabolites depends on the pennation angle. For this reason, Paolo Cerretelli's team has also been involved in the study of skeletal muscle architecture. It has been demonstrated for example by ultrasonography that the pennation angle of the gastroenemius medialis changes during contraction (Narici et al. 1996). Moreover, again in the gastrocnemius medialis, the pennation angle increases from newborn to adolescence (Binzoni et al. 2001). Another interesting observation was that the pennation angle is linearly related to muscle thickness (Fig. 7). In other words, changes in muscle cross-sectional area induced by training may influence the pennation angle and thus η. This will also be the last example of the experimental puzzle generated by the bioenergetic approach.

"Grazie Maestro"

The present paper represents only a modest snapshot of the scientific activity generated by Paolo Cerretelli's enthusiasm. His maxim has always been, and still is, "first define the physiological problem and then find the technique". This is also the reason why in his laboratory new techniques are constantly appearing and disappearing. The main scientific motors driving this work were muscle physiology and the bioenergetic model. To be exhaustive, in the present *European Journal of Applied Physiology* volume you will find a more comprehensive description of the different physiological domains in which Paolo Cerretelli has been involved for many years. Actually, my first concern in writing this present contribution has been not to try to reproduce a historical list of references that may be found in any medical database. It seemed to me, that the most interesting characteristic of Paolo Cerretelli's scientific activity has been the capacity to generate a huge number of ideas, that were often then developed independently by his pupils. Many of these pupils today occupy important places in the scientific world or in society. There is no doubt that this is also one of the important contributions of Rodolfo Margaria's heir and in this case I can only finish by saying: "Grazie Maestro".

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